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PRINCIPAL INVESTIGATOR: Stephanie Gaillard
Donald P. McDonnell, Ph.D.

CONTRACTING ORGANIZATION: Duke University
Durham, North Carolina 27710

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13. ABSTRACT (Maximum 200 Words) Estrogen-related receptors (ERRs) are orphan members of the nuclear receptor superfamily of ligand-dependent transcription factors. Unlike estrogen receptors (ERs), ERRs are unresponsive to natural estrogens and activate transcription constitutively, interacting with required coactivators and DNA-binding elements in the absence of ligand. ERR α has been shown to 1. activate the transcription of pS2, a breast cancer marker, 2. be unresponsive to hormonal therapies, i.e. Tamoxifen, and 3. correlate with unfavorable prognosis in breast tumors. Thus ERR α could be a predictive marker of breast tumor hormone responsiveness and may be a suitable therapeutic target for hormone resistant breast cancers. Therefore, understanding the convergence of the ER and ERR signaling pathways is critical to clarifying the role of ERRs in modulating hormone response.					
Without specific ligands to modulate ERRs, studies of the physiological contributions of these receptors has been limited. We proposed to develop peptide antagonists to specifically inhibit the transcriptional activity of ERRs by blocking receptor-cofactor interactions. Peptides were identified by phage display screening that are capable of inhibiting ERR-mediated transcription without disrupting the transcriptional activity of other nuclear receptors, e.g. ER α and ER β , validating the use of these peptides as specific ERR antagonists. In addition, using these peptides we have identified key regulatory surfaces on the ERR protein that permit the interaction of the receptor with previously validated ERR-coactivators.					
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Introduction

Estrogens and estrogen receptors (ERs) serve a critical function in the development and progression of breast cancer. Indeed, hormone receptor status is an important prognostic indicator of survival and hormone therapy is the first line approach to the treatment of ER positive breast cancers. However, within five years of treatment, most tumors will develop resistance to hormone therapies such as Tamoxifen. Thus, an understanding of the regulation of estrogen production and ER activity is essential to the development of better drug treatments for breast cancer. Estrogen-related receptors (ERRs) are orphan members of the nuclear receptor superfamily of ligand-dependent transcription factors. These receptors share sequence similarities with the ERs, however unlike most nuclear receptors, ERRs activate transcription constitutively, interacting with required coactivators and DNA-binding elements in the absence of ligand. Recently, ERR α has been shown to 1. regulate the local production of estrogen in breast cancer cells (Chen 2002; Yang 1998) and the expression of pS2, a breast cancer marker (Lu 2001b), 2. indirectly regulate ER activity by regulating the expression of ER cofactors (Sanyal 2002), 3. be insensitive to traditional hormone therapy, i.e. Tamoxifen (Coward 2001; Tremblay 2001), and 4. correlate with poorer prognosis in breast tumors (Ariazi 2002).

Because there are no specific ligands for ERRs, it has been difficult to study the role of these receptors in breast cancer. Importantly, without a ligand, the interaction between ERRs and coregulators may be the only step necessary to regulate their activity. Therefore, we set out to develop peptide antagonists that can specifically interact with and modulate the transcriptional activity of ERRs by blocking receptor-cofactor interactions. To do this, baculovirus-expressed hERR α was purified and used to screen seven combinatorial peptide libraries. Several peptides were identified that interact specifically with ERRs and not with a panel of 12 other nuclear receptors. Most importantly, these peptides are capable of inhibiting ERR-mediated transcription without disrupting the transcriptional activity of other nuclear receptors, e.g. ER α and ER β , validating the use of these peptides as specific antagonists of ERR-mediated transcription. Using these peptides we have identified key regulatory surfaces on the ERR protein that permit the interaction of the receptor with previously validated ERR-coactivators. Differences were identified in the requirement for inhibition of coactivation by PGC-1 and the SRC family of coactivators. Mutations in the activation function domain (AF-2) of ERR α confirm that PGC-1 α and SRC do not interact with the same surface of the receptor. Thus to date, these studies have given us a better understanding of the key mechanism regulating the activity of ERRs, the interaction of the receptor with coactivators. Current efforts are on developing systems for expressing the peptide antagonists in cells. This will allow progression to the next phase of the project, the identification of ERR target genes.

Two specific objectives were outlined in the statement of work to be completed in the first year of this grant. The two centered on the use of combinatorial peptide phage display to map protein-protein interaction surfaces on ERRs and develop ERR interacting peptides into peptide antagonists for use in elucidating ERR biology. We have completed these goals and consider the program to be ahead of the original plan. The primary objective in the coming year is to use the ERR peptide antagonists to regulate ERR function in cells.

Task 1 Identification of peptides that interact with ERR using phage display technology. (Months 1-8)

Human ERR α and rat ERR β plasmids were obtained from R. Evans, LaJolla, CA. Human ERR γ was cloned by RT-PCR from a human brain cDNA preparation (Clontech). To date, all efforts have focused on the expression and purification of ERR α . Baculovirus expressed His-tagged full length ERR α has been successfully purified from SF9 insect cells to an estimated >80% purity by Coomassie stained PAGE-gel. The His-tag can be removed post purification by cleaving with an rTEV-protease resulting in purified untagged ERR α . Recently we have also used another vector (DW464) to create a baculovirus that produces a biotin-tagged ERR α protein in greater quantities. The functionality of purified ERR α was tested in an ELISA assay. Purified tagged- and untagged-ERR α is capable of binding an ERE and is also capable of binding the NR box of SRC-1. Purified protein must meet both of these standards to be considered functional protein.

To date, seven peptide-expressing phage libraries have been screened through three rounds of panning and individual peptides have been analyzed for their ability to bind ERR α . Analysis of individual peptides from these seven libraries has been completed. A total of 175 peptides were examined and of these 126 (72%) interacted with ERR α . These peptides were brought forward for analysis in cell based assays (see below).

Task 2 Development of ERR interacting peptides into high affinity peptide antagonists and assessment of the efficacy of ERR peptide antagonists in cell-based assays. (Months 9-14)

Individual peptide-expressing phage were isolated and cloned into a mammalian expression vector to facilitate the analysis of individual peptides via a mammalian-two-hybrid assay. The peptides were initially screened for their ability to bind to the three isoforms of ERR (collectively referred to as ERR unless the specific isoform is specified), ER α , and ER β . All peptides were able to interact with each isoform of ERR though there were some differences in relative abilities to bind. Whether these variabilities actually reflect differences in receptor conformation and ability to recruit coactivators is not yet known. The first criteria for selection of peptides included interaction with ERR α but no interaction with ER α or ER β . Of all the peptides that interacted with ERR α , 37 peptides (29%) did not also interact with either ER α or ER β . These peptides were then tested for their ability to interact with a panel of 12 additional NRs. Five peptides appeared to bind specifically to ERR with minimal binding (<10%) to other receptors tested.

Peptides that were specific for ERR or that showed a selective preference for ERR (bound to fewer than three other receptors) were sequenced and their sequences analyzed. Interestingly all peptides contained an LxxLL motif, a motif previously shown to be important for nuclear receptor-coactivator interactions. An analysis of the sequences flanking the core LxxLL motif identified three major sequence clusters (Table I). Two of the three clusters are similar to sequences previously identified

through phage display screening of ER α (Chang 1999). The studies with ER α defined three classes of ER α interacting peptides. Class I peptides contain a conserved serine at the -2 position and a positively charged residue (R) at the -1 position. Class II peptides have a proline occupying the -2 position and a hydrophobic leucine (L) or isoleucine (I) at the -1 position. Class III peptides share a conserved serine (S) or Threonine (T) at the -2 position followed by a hydrophobic leucine (L) or isoleucine (I) at the -1 position. In our ERR α screens, only one peptide was found that was similar to the class I peptides. However, the majority of peptide sequences fell into either class II or class III. In addition, we have identified another class of peptides, Class IV peptides, which contain a glutamic acid (E) at the -1 position.

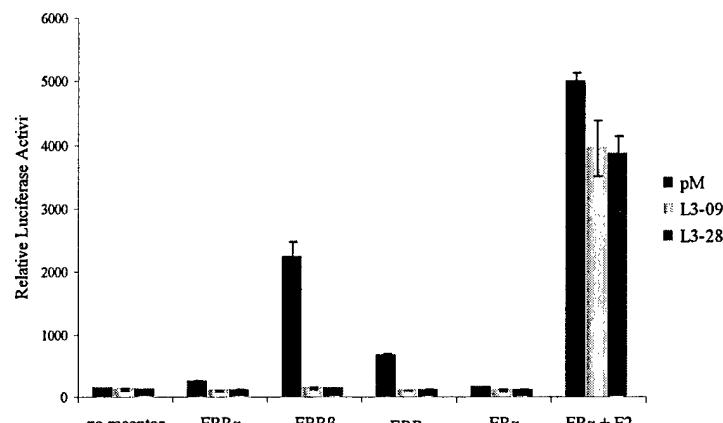
Table I: Classification of NR binding peptides

Class I: (+)LxxLL
Class II: P Φ LxxLL
Class III: (S/T) Φ LxxLL
Class IV: xELxxLL

Four classes of peptides identified by phage display binding to nuclear receptors based on the amino acid residues present at the -1 and -2 positions of the LxxLL motif. Basic amino acids are represented by (+), and hydrophobic residues are depicted by (Φ).

The five ERR-specific peptides were brought forward for further analysis. The peptides were tested for their ability to antagonize the constitutive activity of ERRs in a transcriptional interference assay. Initial studies were conducted on the synthetic minimal promoter 3xERE-TATA-Luc. Two of the five peptides, L3-09 and L3-28, appeared to inhibit constitutive ERR activity to the highest degree. For example in Figure 1, the transcriptional activity of ERR (alpha, beta, and gamma) is inhibited by greater than 90% with either peptide. The transcriptional activity of ER α was only minimally affected by addition of the peptides.

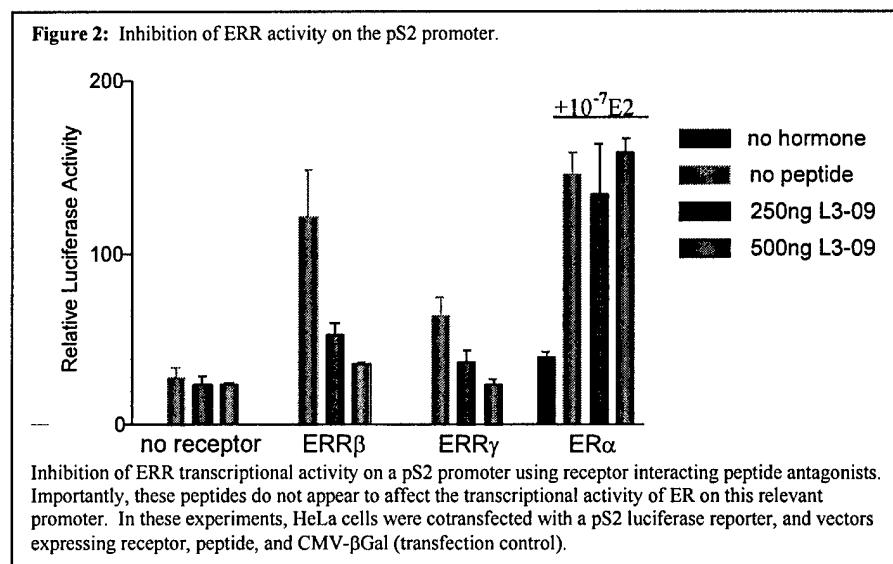
Figure 1: Peptides inhibit ERR transcriptional activity in transfected cells.



Peptides are able to inhibit constitutive ERR transcriptional activity on a synthetic ERE Luciferase reporter (3xERE-Luc). In these experiments, HeLa cells were cotransfected with 3x-ERE-Luc, and vectors expressing receptor, peptide, and CMV- β Gal (transfection control).

Task 3 Evaluation of the biological consequences of blocking ERR-mediated transcription on ERR- and ER-regulated genes. (Months 15-24)

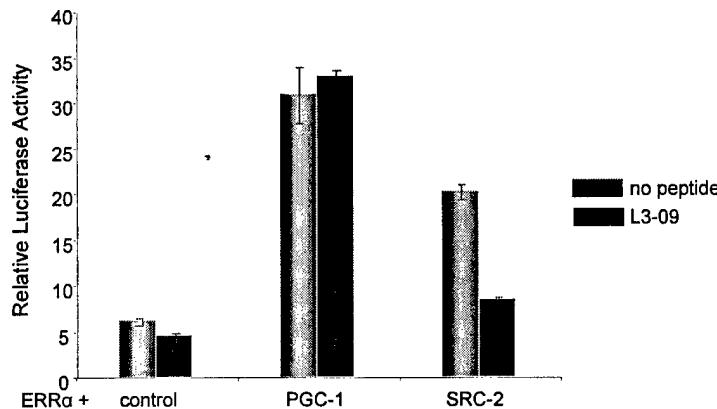
Encouraged by the successful development of peptide antagonists for ERR, we have progressed to part one of task three of the proposal: studying the effect of inhibition of ERR on known target genes. We examined the ability of the peptides to inhibit the constitutive transcriptional activity of ERRs on a pS2-luciferase reporter, a known ERR regulated gene. Similar to the data described above, the peptides were able to specifically inhibit ERR β and ERR γ activity without affecting ER α activity. An example is shown in Figure 2, in which the L3-09 peptide potently interferes with the ability of the ERRs to activate transcription on the pS2 promoter while leaving ER α activity untouched proving that the peptides can specifically target ERR-mediated transcriptional activity.



Previous reports (Lu 2001a) as well as our own data have shown that ER α is only able to activate the pS2 promoter when cotransfected with the p160 family of coactivators (SRC-1, GRIP-1, ACTR). There are two known families of coactivators that interact with the ERRs: the p160s and PGC/PERC. Given the peptide's LxxLL motif along with data showing that the peptides do not interfere with the receptor's ability to bind DNA binding elements within promoters (data not shown), we hypothesized that the peptides prevented coactivator recruitment reducing receptor activity. In order to test the ability of the peptides to compete with coactivators for the receptor, ERR, coactivator, and pS2-Luc reporter were transfected along with the peptide or a control. Surprisingly, the peptide was able to inhibit coactivation by p160 coactivators but did not affect PGC-1 α coactivation (Figure 3). This suggested that the coactivators may interact with different regions of the receptor.

Recent studies (Huss 2002; Schreiber 2003) have identified the AF-2 region of the receptor as the interaction site of PGC-1 α with ERR. However, previous work in our laboratory had implicated the hinge region along with the AF-2 pocket of ER α as important PGC-1 α interaction domains (Tcherepanova 2000). Given the results above, we hypothesized that the PGC-1 α /ERR α interface similarly required regions outside of the AF-2 pocket. In order to test this hypothesis, mutations were made in the AF-2 domain of ER α to determine its importance in

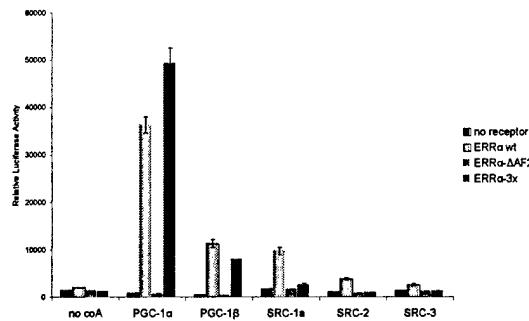
Figure 3: Peptides do not inhibit coactivation by PGC-1.



The addition of coactivators enhances the activity of ERR in a transcriptional assay. Previous studies suggested that all coactivators interacted with ERR in a similar manner through the AF-2 domain of the receptor. Surprisingly, while the peptides were capable of inhibiting coactivation by the p160 family of coactivators, they did not affect the potentiation by PGC-1, another validated ERR coactivator. This suggested that the two coactivator types interacted differently with the receptor. HeLa cells were cotransfected with a pS2 luciferase reporter, and vectors expressing receptor, peptide, coactivator, and CMV- β Gal (transfection control).

coactivator recruitment. A truncated ERR α was generated which lacks the AF-2 region (ERR α - Δ AF2). In addition, three point mutations were made in ERR α 's AF-2 (ERR α -3x). These mutations are analogous to mutations previously generated in ER α and known to interfere with the charge clamp of AF-2 thus preventing the ability of p160 coactivators to bind ER α . As expected removal of the AF-2 (ERR α - Δ AF2) prevented the recruitment of any coactivator (figure 4), consistent with previous reports (Huss 2002; Schreiber 2003). However, AF-2 mutation (ERR α -3x) selectively prevented coactivation by the p160s while leaving PGC-1 α coactivation intact. While it is possible that the other residues in the AF-2 of the ERR-3x mediate PGC-1 α recruitment, it appears that the PGC-1 α /ERR interface is likely also dependent on a region outside of the AF-2, analogous to the ER α /PGC-1 α complex (Tcherepanova 2000) and the results seen in the peptide interference assays. Mutation of the other amino acids in the AF-2 along with constructs in which the hinge region of ERR α is removed will help to define the important interaction sites.

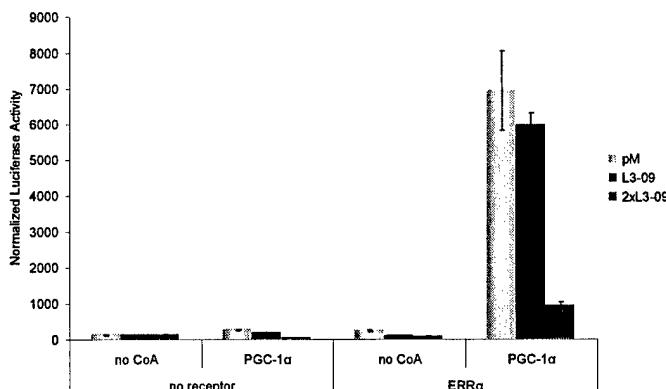
Figure 4: AF-2 point mutations selectively prevent p160 coactivation



Mutations within the AF-2 domain that neutralize the "charge clamp" of helix 12 selectively interfere with p160 coactivation while leaving coactivation by PGC-1 α intact, suggesting that while the AF-2 domain is required for PGC-1 interaction, the positively charged pocket formed by helix 12 is not necessary. HeLa cells were cotransfected with a pS2 luciferase reporter, and vectors expressing receptor, peptide, coactivator, and CMV- β Gal (transfection control).

Analyses of the binding profile of the peptides with the ERR α mutations showed that the peptides all have a profile similar to SRC family members (data not shown) suggesting that this may explain the peptides inability to inhibit PGC-1 α activity. However, another reasonable explanation may be that the affinity of the peptides is below the affinity of PGC-1 α for the receptor. Thus, the peptides are not strong enough to prevent PGC-1 α /ERR interactions. To test whether a peptide of increased affinity could disrupt coactivation by PGC-1 α , we designed a peptide that contains two copies of the L3-09 peptide separated by sequences adapted from those found in PGC-1 α between LxxLL2 and LxxLL3, the two nuclear receptor interacting regions of the coactivator. Two tandem copy peptides of this type have previously been shown to be more effective inhibitors of receptor activity while maintaining specificity (Chang 1999). The 2xL3-09 peptide was able to decrease the potentiation by PGC-1 α , suggesting that PGC-1 α binds to ERR α with a higher affinity than SRC-2 (Figure 5). In addition, these data suggest that cooperativity between the LxxLLs plays an important role in PGC-1 α binding. Additional studies are underway to further define the coactivator:receptor interaction.

Figure 5: PGC-1 α coactivation of ERR α can be disrupted by two copy peptides



HeLa cells were cotransfected with a 3xERE-TATA-luciferase reporter, and vectors expressing receptor, peptide, coactivator, and CMV- β Gal (transfection control).

Currently we are creating an adenovirus designed to overexpress one of the peptide antagonists. Preliminary studies have shown that the adenovirus produces the peptide and that the virus-produced peptide is able to interact with ERR in a mammalian-two-hybrid assay. Transcriptional interference assays are underway with this adenoviral peptide. In addition, a stable MCF7 cell line has been engineered to express the ERR antagonist in an inducible manner. Future experiments with these cells will include mammalian two-hybrids to test peptide-ERR interaction and transcriptional interference assays. The development of these systems to regulate ERR activity will enable our progress in the next phase of this project, the identification and characterization of ERR target genes.

Key Research Accomplishments

- Purification of functional human ERR α protein expressed in a baculovirus system
- Identification of peptides that interact with ERRs through phage display screening of random peptide libraries
- In cell-based assays, identification of peptides which preferentially bind ERRs as compared to 12 other nuclear receptors
- Validation of the use of peptides as specific peptide antagonists (inhibition of ERR activity on the pS2 promoter)
- Identification of a differential interaction of coactivators with ERR α ; the requirement of the charge clamp for SRC coactivators to potentiate ERR activity
- Enhancement of inhibition by two copy peptides
- Development of systems for peptide antagonist expression: adenoviruses expressing peptides and a MCF7 cell line expressing the peptide under an inducible promoter.

Reportable Outcomes

- Presentation of research at several conferences and seminars:
 - Medical Scientist Training Program Symposium *June 2003*
 - Endocrine Society Conference *July 2003*
 - Department of Pharmacology and Cancer Biology Annual Retreat *September 2003*
 - Biological Sciences Graduate Student Research Day *November 2003*
 - Keystone Symposium: Nuclear Receptors (Steroid Sisters) *March 2004*
 - Duke University Graduate Student Research Day *March 2004*
 - Sex and Gene Expression Conference *March 2004*
 - Medical Scientist Training Program Symposium *March 2004*
 - Cancer Biology Student Seminar Series *March 2004*
- Awards received based on research:
 - Endocrine Society Travel Award *July 2003*
 - Best Poster Award, Biological Sciences Graduate Student Research Day *November 2003*
 - Florence P. Hasseltine Award for the Outstanding Presentation by a New Investigator in Sex-Based Biology at the Fifth Annual Conference on Sex and Gene Expression Conference *March 2004*
- Entered into candidacy for a Ph.D. through the Department of Pharmacology and Cancer Biology, Duke University *November 2003*
- Reagents generated through this project:
 - Expression vectors for ERR specific peptide antagonists, including two-copy peptides
 - Adenovirus expressing peptide antagonist L3-09
 - MCF-7 cell line expressing peptide antagonist L3-09
 - ERR mutations: ERR α - Δ AF2, ERR α -3x

Conclusions

The goal of our project is to understand the role of ERRs in regulating ER function in breast cancer cells. ERRs have traditionally been difficult to study because they are orphan nuclear receptors and there are no ligands which could be used to specifically modulate receptor activity. Through this project, we have developed the first peptide antagonists for use with an orphan nuclear receptor. These peptides are a powerful reagent which can be used to specifically target ERR activity allowing us to study the effect these receptors have on ER activity in breast cancer. This approach could easily be developed to target other otherwise intractable orphan nuclear receptors.

As a result of our current work, we have a better understanding of binding of coactivators to ERR, the key regulatory mechanism determining the activity of the receptor. We have identified the critical regions of the receptor important for coactivator binding and defined differential binding requirements between coactivator families. In addition, we have shown that it is possible to regulate ERR transcriptional activity on known target genes. The development of potent expression systems to enhance delivery of the peptides to the cells will allow us to probe the physiological function of the receptor and identify new target genes. It is the elucidation of ERRs' mechanisms of regulation of estrogen-responsive genes that we anticipate will enable us to advance our comprehension of breast cancer progression.

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